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Function of the Outer Membrane of *Escherichia coli* as a Permeability Barrier to Beta-Lactam Antibiotics

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On the basis of a simple theoretical model, the ease of penetration of β-lactam antibiotics through the outer membrane of *Escherichia coli* was measured. The cell envelope was found to act as a diffusion barrier to both penicillins and cephalosporins. The validity of the model and the cooperative action of cell-bound β-lactamase and outer membrane were further verified by comparing calculated and experimentally determined velocities of β-lactam hydrolysis by intact cells and sonically treated cell suspensions. The results showed good correspondence at five different antibiotic concentrations. Similar conclusions could be drawn from a comparison of β-lactam concentrations on both sides of the outer membrane, calculated from enzyme kinetic measurements and minimal inhibitory concentrations for both a β-lactamase-producing *E. coli* and its enzyme-negative variant. in the case of benzylpenicillin and cephalothin, however, no correspondence was found. The joint action of several parameters determining the efficacy of penicillins and cephalosporins against β-lactamase-producing *E. coli* is discussed.

Escherichia coli can carry extrachromosomal resistance determinants (R factors) mediating constitutive synthesis of β-lactamase (EC 3.5.2.6). The production of this enzyme renders the organisms less susceptible to the lethal action of penicillins and cephalosporins and may even lead to complete resistance to these antibiotics (14, 15, 17).

Whereas gram-positive β-lactamase-producing organisms liberate their enzyme into the surrounding medium, the β-lactamases of gram negative bacteria are compartmentalized (4, 15). The fact that these enzymes can be totally released from *E. coli* by osmotic shock (8, 9) demonstrates that they are attached loosely to the cytoplasmic membrane or localized in the periplasm.

Both β-lactamases and the outer membrane are important factors in the resistance of *E. coli* to penicillins and cephalosporins (1-3, 18). Conclusions regarding the ease of penetration of these antibiotics through the outer membrane, however, have mainly been drawn from comparisons of rates of hydrolysis by intact cells and sonically treated cell suspensions (6, 15). This determination alone is not sufficient to measure outer-membrane permeability.

Given a periplasmic localization of β-lactamase and assuming that both penicillins and cephalosporins reach their targets by a simple diffusion process, we have developed a different approach to measuring the role of the outer membrane of *E. coli* as a permeability barrier to β-lactam antibiotics. Experimental evidence is presented to show that the difference in β-lactam concentration on either side of the outer membrane depends not only on the permeability of the outer membrane, but also on the kinetic characteristics of the cell-bound periplasmic β-lactamase.

MATERIALS AND METHODS

Bacterial strains, growth conditions, media and buffers. *E. coli* 205 (serotype O55) and its R-factor-carrying variant *E. coli* 205 ($R^{\dagger}_{TEM}$) were used in all experiments. Both are standard screening strains from our laboratories and are designated as *E. coli* (R^-) and *E. coli* (R^+), or simply as R^- and R^+ in the following text. The TEM R factor was transferred to *E. coli* 205 from *E. coli* TEM ($R^{\dagger}_{TEM}$) (4). The media used were brain heart infusion and brain heart infusion agar (BBL, Cockeysville, Md). Cells were grown at 37°C. Phosphate buffer was 0.067 M Sörensen buffer, pH 7.0.

β-Lactamase assay. β-Lactamase activity was assayed by a modification of the micro-iodometric method (12). Substrate was preincubated in a water bath for 5 min to 37°C, and the reaction was then initiated by adding enzyme solution (intact cells or sonically treated cell suspension; see below). Hydrolysis was stopped by adding up to 1 ml of reaction mixture to 1 ml of starch-iodine reagent, and the

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total sample volume was made up to 2 ml with phosphate buffer if necessary. Starch-iodine reagent was prepared by mixing 100 μl of 0.08 M iodine–3.2 M potassium iodide with 80 ml of 0.25 M sodium tungstate in 1 N acetic acid and then adding 20 ml of 2% (wt/vol) soluble starch which had been dissolved in 1 N acetic acid by gentle boiling for 2 to 3 min. In all assays, we ran controls of identical composition but to which the enzyme had been added to the substrate after the admixture of the starch-iodine reagent. Absorbance was measured at 623 nm 20 min after the reaction had been stopped. To convert rates of decolorization of the blue starch-iodine complex into enzyme reaction velocities, the iodine consumption of each substrate was determined experimentally after complete hydrolysis of penicillins or cephalosporins with TEM-type β-lactamase or β-lactamase from *Aerobacter cloacae* P 99 (7), respectively.

Determination of velocities of β-lactam hydrolysis by intact cells and sonically treated suspensions. Exponentially growing cultures of *E. coli* (R^+) were harvested by centrifugation for 15 min at 5,000 × *g* at 2°C and resuspended at 10-fold the original density in ice-cold phosphate buffer. A small part of this suspension was used directly in assaying the β-lactamase activity of intact cells (v_{intact}). The rest of the cells were converted to spheroplasts (13), and complete liberation of β-lactamase was achieved by short ultrasonic treatment of the spheroplasts at 0°C with a Branson B-12 Sonifier (Branson Sonic Power Co., Danbury, Conn.). This suspension was then used to measure the velocity of hydrolysis by sonically treated cell suspensions ($v_{sonicated}$).

Enzyme kinetic parameters. Michaelis constants (K_m) and maximal rates of substrate hydrolysis (V_{max}) were determined with enzyme from a sonically treated suspension of *E. coli* 205 (R^+) prepared as described above. Both parameters were calculated from a plot of $[S]/v$ versus $[S]$ (Woolf plot).

Measurement of MICs. Minimal inhibitory concentrations (MICs) were determined in a short-term assay with a small inoculum. Exponential-phase cultures were diluted to a density of 10^3 cells/ml. Portions of these cultures were prewarmed for 5 min to 37°C, and the MIC determination was started by adding increasing concentrations of antibiotic. Viable-cell counts were made at the beginning and after 2 h of incubation by directly plating 0.2 ml from each culture on 20-ml agar plates and counting the colonies after 18 h of incubation at 37°C. The MIC was defined as the concentration at which neither an increase nor a decrease in the viable-cell count occurred during 2 h of incubation. It was determined graphically by interpolation.

Antibiotics and reagents. The cephalosporins and penicillins investigated were cephacetrile (Ciba-Geigy Ltd., Basel, Switzerland), cephaloridine (Glaxo Ltd., Greenford, Middlesex, U.K.), cephalothin (Eli Lilly & Co., Indianapolis, Ind.), cefazolin (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan), benzylpenicillin (Novo Industri A/S, Copenhagen, Denmark), ampicillin (Beecham Research Laboratories, Betchworth, Surrey, U.K.), and two experimental cephalosporins synthesized in the laboratories of Ciba-Geigy Ltd., Basel: C 49,288 [7-cyanacetamido-3-(1-methyltetrazole-5-ylthio)methylceph-3-em-4-carboxylic acid, sodium salt] and C 49,753 [7-cyanacetamido-3-(1,3,4-thiadiazole-5-ylthio)methylceph-3-em-4-carboxylic acid, sodium salt].

The soluble starch used was starch-gel according to Smithies (Serva Feinbiochemica GmbH & Co., Heidelberg, Germany).

RESULTS

Postulates made for determining the permeability parameter *C*. Our general assumptions were the following. (i) The outer membrane of *E. coli* is a diffusion barrier to both penicillins and cephalosporins and is characterized by a permeability parameter *C*. (ii) β-Lactam antibiotics reach the site of β-lactamase action in the periplasm by passive diffusion, not by active transport (10, 11). (iii) K_m and V_{max} are the same both for cell-bound β-lactamase in the intact cell and for free enzyme in sonically treated suspensions. (iv) At a given antibiotic concentration outside the β-lactamase-synthesizing cells, S_0, a steady state, is rapidly established at which the rate of antibiotic diffusion and the velocity of β-lactam hydrolysis are equal. At the steady state, the antibiotic concentration inside the outer membrane is S_e. It is determined by the four parameters S_0, K_m, V_{max}, and *C*. Hence:

$$\text{steady state: } C(S_0 - S_e) = \frac{S_e \cdot V_{max}}{S_e + K_m} \quad (1)$$

and

$$S_e = 0.5 \left[S_0 - K_m - \frac{V_{max}}{C} + \sqrt{\left(-S_0 + K_m + \frac{V_{max}}{C}\right)^2 + 4S_0K_m} \right] \quad (2)$$

Experimental determination of the permeability parameter *C*. Velocities of β-lactam hydrolysis by intact cells and by sonically treated cell suspensions of *E. coli* (R^+) were measured at a 0.1 mM substrate concentration. The antibiotic concentration S_e on the inner side of the outer membrane was then calculated from $v_{intact}/v_{sonicated}$ and from K_m and V_{max} determined with sonically treated suspensions:

$$S_e = (v_{intact} \cdot K_m)/(V_{max} - v_{intact}) \quad (3)$$

From these data, the relative values for the diffusion parameter *C* were obtained from equation (1). Five of the six cephalosporins tested had very similar *C*-values (Table 1). On the other hand, the barrier function of the outer membrane was more pronounced for cephalothin and the two penicillins. Comparison of the

partition coefficients given in the last column indicates that these three antibiotics are also the most lipophilic β-lactams in the whole group tested.

Comparison of calculated and experimentally determined values of $v_{intact}/v_{sonicated}$ at different antibiotic concentrations S_0. According to our assumptions, S_e and consequently also $v_{intact}/v_{sonicated}$ depend not only on K_m, V_{max}, and C, but also on the concentration S_0 at which these two velocities are measured. If these assumptions are valid, then $v_{intact}/v_{sonicated}$ must be practically constant at low values of S_0, and both velocities should become equal at high antibiotic concentrations. Table 2 gives a comparison of $v_{intact}/v_{sonicated}$ calculated and determined experimentally at five different values of S_0. The calculations are based on the values of K_m, V_{max}, and C in Table 1 and on equation (2) above. In general, there was a good correspondence between predicted and experimentally measured data. Benzylpenicillin and ampicillin were only compared at 5 mM concentrations, and v_{intact} was corrected for hydrolysis by leaked enzyme. Leakage of even very low amounts of β-lactamase makes it very difficult to determine v_{intact} accurately with these two antibiotics, since the differences in the antibiotic concentrations on either side of the outer membrane are large and a small quantity of free enzyme can hydrolyze more of these two substrates than the total cell-bound β-lactamase active at a much lower concentration S_e.

Further experimental verification of our basic assumptions. It can reasonably be assumed that the susceptibility of the cell wall-synthesizing enzymes, the targets of β-lactam antibiotics, is not influenced or altered by the presence of β-lactamase. The MIC for a β-lactamase-producing *E. coli* can thus be interpreted as a concentration, S_0, which is reduced by the activity of the hydrolyzing enzyme inside the outer membrane to an inner concentration, S_e, corresponding to the MIC determined with a β-

TABLE 1. *Determination of antibiotic concentration S_e at the site of β-lactamase action and of the diffusion parameter C at a concentration S_0 = 0.1 mM*

Antibiotic	$\frac{v_{intact}}{v_{sonicated}}$	K_m (μM)	V_{max}[a]	S_e (μM)	C (×10⁻⁴)	P[b]
Cephacetrile	0.58	1,400	100	57	8.9	0.021
Cephaloridine	0.22	1,050	650	21	16	0.124
Cephalothin	0.07	300	70	5.4	1.3	0.259
Cefazolin	0.34	450	80	30	7.0	0.037
C 49,288	0.55	1,050	145	53	15	0.012
C 49,753	0.39	450	115	34	12	0.020
Benzylpenicillin[c]	0.034	20	580	0.7	1.9	0.6
Ampicillin[c]	0.04	40	650	1.6	2.5	0.159

[a] Relative values; V_{max} of cephacetrile = 100.
[b] Partition coefficient in iso-butanol–0.02 M phosphate buffer (pH 7.4)–0.9% (wt/vol) NaCl; experiments done at 37°C.
[c] $v_{intact}/v_{sonicated}$, S_e, and C determined at S_0 = 1 mM.

TABLE 2. *Comparison of calculated and experimentally determined values for $v_{intact}/v_{sonicated}$ at five different antibiotic concentrations S_0*

Antibiotic	$v_{intact}/v_{sonicated}$ at S_0 value of:				
	0.02 mM	0.05 mM	0.5 mM	1 mM	5 mM
Cephacetrile	0.55[a]/0.61[b]	0.56/0.58	0.67/0.61	0.75/0.72	0.96/0.94
Cephaloridine	0.21/0.17	0.21/0.19	0.30/0.30	0.38/0.44	0.82/0.97
Cephalothin	0.06/0.06	0.06/0.08	0.14/0.14	0.23/0.19	0.80/0.54
Cefazolin	0.29/0.32	0.31/0.32	0.54/0.52	0.72/0.72	0.98/1.1
C 49,288	0.50/0.52	0.53/0.55	0.67/0.66	0.77/0.77	0.97/1.02
C 49,753	0.33/0.27	0.34/0.33	0.58/0.54	0.76/0.77	0.98/1.04
Benzylpenicillin	ND[c]	ND	ND	ND	0.16/0.28
Ampicillin	ND	ND	ND	ND	0.19/0.11

[a] Calculated with K_m, V_{max}, and C from Table 1.
[b] Measured experimentally; mean of at least two determinations.
[c] ND, Not determined.

TABLE 3. *Comparison of the quotients MIC R⁻/MIC R⁺ with calculated values for S_e/S_0*

Antibiotic	MIC R⁻/MIC R⁺	S_e/S_0 (calculated)
Cephacetrile	0.5	0.5
Cephaloridine	0.1	0.2
Cephalothin	0.4	0.05
Cefazolin	0.4	0.25
C 49,288	0.8	0.5
C 49,753	0.6	0.3
Benzylpenicillin	0.033	0.001
Ampicillin	0.006	0.002

lactamase-negative variant. The ratio of the MICs obtained with the R⁻ and R⁺ strains of *E. coli* 205 should therefore correspond to the S_e/S_0 value of the β-lactamase-producing cell calculated at S_0 equal to the MIC of the R⁺ strain. This assumption, however, is correct only as long as the antibiotic concentration S_0 remains constant throughout the MIC determination. In our case, this condition was fulfilled by using a short-term assay at low cell densities. A comparison of the experimentally determined MIC R⁻/MIC R⁺ quotients and the S_e/S_0 values obtained by calculation is given in Table 3. Again, there was a relatively good correspondence between the calculated and measured data, providing further evidence of the validity of our model. The reasons for the large difference between the results for benzylpenicillin and cephalothin, however, are unknown. It is possible that the barrier function of the outer membrane is altered during the MIC determination.

DISCUSSION

The permeability of the outer membrane has been studied in detail by Nikaido (10, 11). He concluded that low-molecular-weight hydrophilic antibiotics diffuse through aqueous pores. The results of the present study agree with this assumption. They clearly show that the outer membrane of *E. coli* is a diffusion barrier for both penicillins and cephalosporins. Its apparently unrestricted penetrability for cephaloridine, described by several authors (4, 6, 16), is certainly explained by the high concentrations they used in measuring the velocity of cephaloridine hydrolysis. The data in Table 1 also indicate that an increase in the lipophilic character of the β-lactam tends to decrease its rate of diffusion.

A simple comparison of the velocities of hydrolysis by intact cells and sonically treated cell suspensions is not enough to indicate the ease of passage of a β-lactam antibiotic through the outer membrane. In a theoretical study of synergism between β-lactam antibiotics, Hamilton-Miller (5) calculated the antibiotic concentration S_e in the periplasm from the three parameters S_0, K_m, and a substrate permeability factor, P, equal to $v_{sonicated}/v_{intact}$. In contrast to our diffusion parameter C, however, this factor P is not a direct measure of outer-membrane permeability. In addition, the results in Table 2 show clearly that P depends on the β-lactam concentration S_0 at which it is determined.

When considering the efficacy of penicillins or cephalosporins against β-lactamase-producing *E. coli*, it should also be noted that all our experiments were made at a practically constant S_0. They reveal the degree of protection that the combined action of the outer membrane and β-lactamase can afford to each individual cell. Against benzylpenicillin and ampicillin these defenses are very efficient, but the cells remain relatively susceptible to most of the cephalosporins tested. In infections with whole populations of β-lactamase-producing *E. coli*, however, the possibility must also be considered that additional protection may be achieved through an enzyme-mediated reduction in the serum or tissue concentration of the penicillin or cephalosporin in question.

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